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TITLE: Molecular Differentiation of Risk for Disease
         Progression: Delineating Stage-Specific Therapeutic
         Targets for Disease Management in Breast Cancer

PRINCIPAL INVESTIGATOR: Maria J. Worsham, Ph.D.
                          Usha Raju, M.D.
                          Gary Chase, Ph.D.
                          Mei Lu

CONTRACTING ORGANIZATION: Henry Ford Health System
                           Detroit, Michigan 48202

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6. AUTHOR(S)
Maria J. Worsham, Ph.D.
Usha Raju, M.D.
Gary Chase, Ph.D.
Mei Lu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Henry Ford Health System
Detroit, Michigan 48202

E-Mail: mworshal@hfhs.org

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13. ABSTRACT (Maximum 200 Words)

Cancer is a highly heterogeneous disease, both morphologically and genetically. A current shortcoming in cancer prognosis and treatment is a lack of methods that adequately address the complexity and diversity of the disease. Genome wide studies can provide molecular characterization or fingerprints of cancer phenotypes linked to clinical information. The aim of this research is to **1a:** identify an informative set of specific genetic alterations that underlie the pathogenesis of disease progression to serve as targets for management of disease at the earliest stages and **1b:** refine stage-specific disease phenotypes by integration of molecular profiles with known risk factors of breast cancer such as reproductive characteristics, medical history, and histologic parameters of breast carcinomas. We will examine 100 cases in each disease stage category of 0, 1, 2, 3, 4 and unknown to evaluate 120 breast cancer associated gene markers distributed throughout the human genome. Molecular fingerprints identified from genome wide studies should delineate patterns of genomic imbalances at the level of stage-specific gene loci, providing a novel index to estimate the extent of genomic abnormality with disease progression. This knowledge should allow the integration of stage-specific therapeutic targets as treatment intervention strategies in the management of breast cancer.

14. SUBJECT TERMS
Disease progression, genome wide, gene loci alterations, stage-specific markers, breast cancer

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INTRODUCTION

Cancer is a highly heterogeneous disease, both morphologically and genetically (1). A current shortcoming in cancer prognostication and treatment is a lack of methods that adequately address the complexity and diversity of the disease. A detailed molecular characterization or fingerprint of cancer is an objective recently made possible by the development of several new high throughput analytical methods. These include techniques for the analysis of DNA, mRNA, and proteins within a cell (2-4). Building databases of detailed molecular information and linking them to clinical information are very attainable goals (5). This approach has the potential to help patients by improving grouping of tumor subtypes, which may enable clinicians to more accurately distinguish prognostic groups, and predict the most effective therapies. Prognostic marker systems based on single parameters have generally proven inadequate. Thus, multiparametric methods, which rely on many pieces of information, are ideally suited to the grouping of tumor subtypes and the identification of specific patterns of disease progression.

A major objective of current cancer research is to develop a detailed molecular fingerprint of tumor cells and tissues that is linked to clinical information. Toward this end, using the Multiplex Ligatable Probe Amplification technique (MLPA, 6), a novel assay recently developed at MRC Holland (Amsterdam) we will interrogate 120 gene loci (Table 1, Study Instruments) altered in breast cancer using a nested case cohort of 600 stage-specific breast cancers drawn from a retrospective cohort of 6000 primary breast cancers.

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6.   MRC Holland: Dr. J. P. Schouten, mrch@bio.vu.nl. Website: www.MRC-Holland.com

Statement of Work

Task 1. Cohort construction, Months 1-24

a: Begin construction of the breast cancer study cohort. We have identified 6000 breast cancer cases in the HFHS system from 1981 through 2000. Drs. Worsham and Chase will select 100 stage-specific breast cancers corresponding to stage 0 (in situ), stage 1, 2, 3, 4 and unknown stage
b: Set up database of study cohort
c: Retrieval of H & E slides for cancer cohort
d: The Pathologist Dr.Raju and the P.I will begin pathology review of the cancer cohort recording histopathological characteristics on the Pathology Cancer Review Form (see study instruments).
e: Data entry of histopathology indicators
f: selection of tumor blocks and sectioning of tissue for microdissection and DNA extraction

Task 2. Molecular Assays   Months 3-34

a: Begin the novel Multiplex Ligatable Probe Amplification(MLPA) assays
b: As DNA becomes available set up molecular worksheets and forms for electronic data entry of molecular data (Teleform)

Task 3. Medical chart abstraction   Months 3-32
a: Begin medical chart abstraction using the Medical record Abstraction Form
b: Data entry of forms into the study database

Task 4. Interim Analyses, Months 18-24

a: Interim statistical analysis of data obtained from molecular, pathology, and medical record abstractions will be performed periodically
b: Annual reports will be written

Task 5. Final Analyses and Report Writing, Months 32-36
a: Final analyses of data form molecular, pathology and medical abstractions will be performed
b: A final report and initial manuscripts will be prepared

PROGRESS (July 1, 2003- June 30, 2004:

KEY RESEARCH ACCOMPLISHMENTS
Task 1 accomplishments:
- **July 1, 2002-June 30th 2003:**
  We have so far acquired a total breast cancer patient database of 5008 validated and verified breast cancer cases. The study cohort of 600 stage-specific breast cancer subjects was derived from this comprehensive patient database. Selection of breast cancer subjects in each of the 6 stages, stage 0, stage 1, stage 2, stage 3, stage 4, and stage unknown was performed by the biostatistician Dr. Gary Chase. Criteria for selection were as follows: 1) age <50 years, Caucasian Americas (CA); age <50 years, African American (AA); 2) age >50 years, CA; age >50 years, AA. A total of 1,244 subjects were obtained as a result of this selection; stage 0=215; stage 1= 225; stage 2= 228; stage 3= 188; stage 4= 179; stage unknown= 209. Further selection of 100 stage-specific cases for equal representation of CA and AA in each of the two age categories, <50 years and > 50 years was achieved in a random fashion by Dr. Gary Chase. Thus, the study cohort of 600 breast cancer subjects, 100 in each of the 6 stages has been completed. **Status: Completed**

July 1, 2002-June 30th 2003
- Data bases of the study cohort have been completed and linked with the Henry Ford Health System Tumor Registry for demographics, histopathology, and clinical information. The latter has been obtained for the entire cohort of 1,244 subjects. **Status: Completed**

July 1, 2002-June 30th 2003:
- The study Pathologist Dr. Raju has completed review of 210 breast cancer subjects
  July 1, 2003-June 30th 2004
  The study Pathologist Dr. Raju has completed review of an additional 209 cases to bring the total cases reviewed to 419. **Status: Completed review of 419/600 breast cancer patients**

July 1, 2002-June 30th 2003
- Pathology Review Form data via electronic Teleform data entry has been entered for 210 study subjects
  July 1, 2003-June 30th 2004
  Pathology Review Form data via electronic Teleform data entry has been entered for an additional 60 patients: **Status: PRF data entry completed for an additional 270 cases.**

July 1, 2002-June 30th 2003

- Tissue block retrieval, sectioning, H & E staining, microdissection, and DNA extraction has been accomplished for 160 subjects

    July 1, 2003-June 30th 2004

    Tissue block retrieval, sectioning, H & E staining, microdissection, and DNA extraction has been accomplished for an additional 110 subjects. **Status: Completed an additional 110 cases for a total of 270 subjects**

Task 2

July 1, 2002-June 30th 2003

- Multiplex Ligatable Probe Amplification (MLPA) assays have been performed for 120 subjects for a total of 309 lesions.

    July 1, 2003-June 30th 2004

    Multiplex Ligatable Probe Amplification (MLPA) assays have been performed for an additional 78 Patients a total of 497 lesions (MLPA reactions). **Status: Completed an additional 78 cases for MLPA for a total of 198 cases and 806 lesions**

Task 3

July 1, 2002-June 30th 2003

- Medical record abstraction has been completed for 120 subjects and entered into the database

    July 1, 2003-June 30th 2004

    Medical record abstraction has been completed for an additional 106 subjects and entered into the database. **Status: Completed an additional 106 subjects for a total of 226 subjects**

REPORTABLE OUTCOMES

Worsham MJ, Sethi S, Qureshi H, Lu M, Tiwari N, Raju U. Molecular differentiation of pleiomorphic lobular carcinoma in situ. ASIP, April 17th, 2004

Molecular differentiation of pleiomorphic lobular carcinoma in situ

Abstract:

While classic LCIS is considered a risk marker for cancer as compared to DCIS, the clinical and biological significance of PLCIS is currently unknown. The predictive ability of a genome wide probe panel to differentiate PLCIS from LCIS and DCIS was examined in a study cohort of 57 patients, 23 PLCIS, 13 LCIS and 21 DCIS. Patient cohort DNA was interrogated for gene loss and gain at 122 gene loci. An interpretation of gene loss or gain was measured as the number of copies in a range of 0 to more than 2 copies, respectively, where 2 (copies) was normal. We tested the difference in mean copy number among tissue categories of LCIS, PLCIS, and DCIS using ANOVA. Of the 10 gene probes selected with overall p-values <0.1, four gene probes, *CDH1* (16q22.1), *PDCD8* (Xq25-26), CASP1 (11q22.2-q22.3), and *PTK2* (8q24) had a difference in means between PLICS and LCIS with p-values <0.05. Of these, *CASP1* differed significantly in means among PLCIS, LCIS and DCIS. To test for correlation among gene probes, an absolute correlation coefficient (ACC) was set in the range of 0 to 1. An ACC of 0.48 was noted between *PTK2* and *TNFRSF1B* (1p36.3), and 0.06 between *CASP1* and *TNFRSF1B*. Further validation of these molecular fingerprints should permit a more robust differential diagnosis of PLCIS aiding in the refinement of this disease phenotype as distinct from other in situ lesions.

Appendices

Manuscript draft: Molecular differentiation of pleiomorphic lobular carcinoma in situ

BACKGROUND

Most breast carcinomas in situ are easily categorized as ductal (DCIS) or lobular (LCIS). However, some carcinoma in situ lesions have indeterminate histological features (CIS-IF) [1, 2]. A pleiomorphic variant
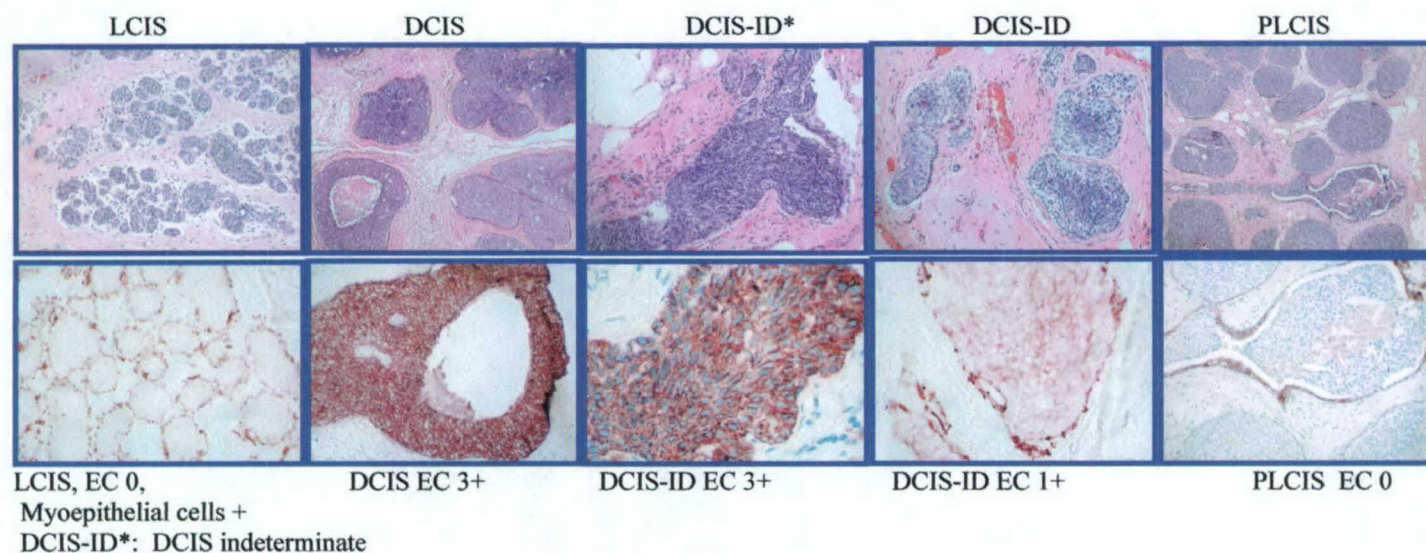
of invasive lobular carcinoma (PILC) is known to be an aggressive variant of invasive lobular carcinoma (ILC) [3]. Its in-situ counterpart, pleomorphic lobular carcinoma in situ (PLCIS), defined by Frost et al. (4) in 1996, has not been fully defined histologically and biologically (4). Moreover, while classic LCIS is considered a risk marker for cancer when compared to DCIS, the clinical and biological significance of PLCIS is currently unknown (4).

PLCIS like PILC is expected to be more aggressive than LCIS. Morphologically it looks similar to intermediate grade DCIS. In the past, because of the histological similarity and associated necrosis, most PLCIS lesions have been diagnosed as DCIS. Treatment strategies are different for different types of breast carcinomas in situ. If a diagnosis of LCIS is made, the patient is followed up for observation (2), whereas a diagnosis of DCIS results in definitive treatment, depending on the extent and grade of DCIS (mastectomy, lumpectomy and radiation therapy or observation alone). Because of the expected aggressive behavior of PLCIS, it is believed that treatment similar to DCIS may be warranted.

Additional methods to adequately aid in the accurate differential diagnosis of in situ breast carcinomas has clinical implications, as current management of classic LCIS versus PLCIS and DCIS is not identical. Gene expression of E-cadherin (EC) provides some degree of lesion sub typing (5-7), **Figure 1**. However, while a negative EC stain can confirm a diagnosis of classic ILC and PLCIS it cannot distinguish LCIS and ILC from PLCIS. High throughput genome wide molecular genetic approaches delineating differences in LCIS, PLCIS and DCIS might help in developing a consensus diagnosis and treatment.

The overall aim of the proposed research is to identify an informative set of genetic alterations that can distinguish the PLCIS disease phenotype from other in situ and invasive breast carcinomas.

Figure 1   : H & E and E-cadherin staining

LCIS	DCIS	DCIS-ID*	DCIS-ID	PLCIS

LCIS, EC 0,   DCIS EC 3+   DCIS-ID EC 3+   DCIS-ID EC 1+   PLCIS EC 0
 Myoepithelial cells +
DCIS-ID*: DCIS indeterminate

METHODS

To assess the ability to molecularly differentiate between carcinoma in situ lesions of PLCIS, LCIS, and DCIS using our genome wide strategy and to ascertain whether there exists a molecular basis for in situ lesion differentiation, the predictive ability of a breast cancer specific 122 gene probe panel was examined in a study cohort of 57 patients. DNA from in situ breast carcinoma patients classified into the three categories of PLCIS, LCIS and DCIS were interrogated for gene loss and gain at 122 gene loci. We selected one carcinoma in situ tissue type per subject (patient) with priorities of tissue selection in the order of PLCIS, LCIS and DCIS. There were 57 patients, 23 (40%) PLCIS, 13(23%) LCIS and 21(37%) DCIS. An interpretation of gene loss or gain was measured as the number of copies in a range of 0 to more than 2 copies, respectively, where 2 (copies) was
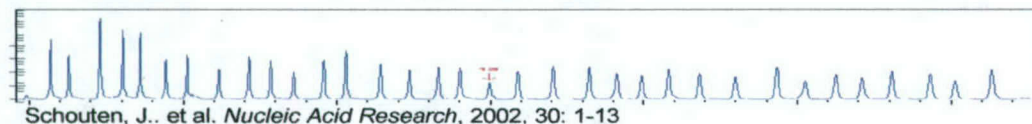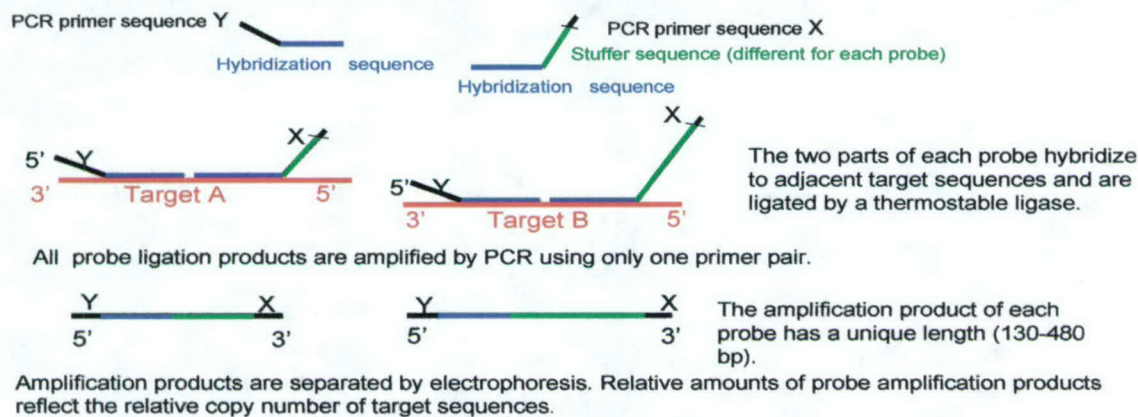
normal. To examine whether the 122 gene probe panel differentiated among in situ tissue categories, for each gene probe, we tested the difference in mean copy number among tissue categories using ANOVA.

The Multiplex Ligation-dependent Probe Amplification Assay (MLPA)

The MLPA assay, recently developed at MRC Holland (Amsterdam, The Netherlands) is a new method for relative quantification of approximately 30-40 different DNA sequences in a single reaction requiring only 20 ng of human DNA. MRC Holland is a privately owned laboratory located within the buildings of Amsterdam's Free University. MLPA techniques are proprietary and currently are under patent procurement. The assay has been successfully used for the detection of deletions and duplications of complete exons in the human *BRCA1*, *MSH2* and *MLH1* genes, detection of trisomies such as Down's syndrome, characterisation of chromosomal aberrations for gains and losses of genes in cell lines and tumor samples, and relative quantification of mRNA's (8, 9). Probes added to the samples are amplified and quantified instead of target nucleic acids. Amplification of probes by PCR depends on the presence of probe target sequences in the sample. Each probe consists of two oligonucleotides, one synthetic and one M13-derived, each hybridizing to adjacent sites of the target sequence. Such hybridized probe oligonucleotides are ligated, permitting subsequent amplification (**Figure 2**). All ligated probes have identical end sequences, permitting simultaneous PCR amplification using only one primer pair. Each probe gives rise to an amplification product of unique size between 130 and 480 bp. Probe target sequences are small (50-70 nucleotides). The prerequisite of a ligation reaction provides the opportunity to discriminate single nucleotide differences. The amplified fragments are separated on a DNA sequencer.

Multiplex Ligation-dependent Probe Amplification (MLPA)

- Denatured genomic DNA is hybridized with a mixture of ~ 40 probes.
- Each MLPA probe consists of two oligonucleotides, one synthetic and one M13-derived.

PCR primer sequence Y
Hybridization sequence
PCR primer sequence X
Stuffer sequence (different for each probe)
Hybridization sequence

5' Y
3' Target A 5'
5' Y
3' Target B 5'

The two parts of each probe hybridize to adjacent target sequences and are ligated by a thermostable ligase.

All probe ligation products are amplified by PCR using only one primer pair.

Y X
5' 3'
Y X
5' 3'

The amplification product of each probe has a unique length (130-480 bp).

Amplification products are separated by electrophoresis. Relative amounts of probe amplification products reflect the relative copy number of target sequences.

Schouten, J.. et al. *Nucleic Acid Research*, 2002, 30: 1-13

Worsham, MJ et al. *Arch of Otolaryngol Head and Neck Surg*, 2003, 129: 702-708

Figure 2

One of the applications of the MLPA method is the detection of chromosomal aberrations in DNA samples from tumors (8, 9). The quantitative nature of the results allows the detection of loss of a gene copy (loss of heterozygosity) without the need for informative heterozygous markers.

We have created and validated a panel of 122 gene probes known to detect altered genes during tumor progression that will address functional categories of: a) growth/apoptosis/repair; b) immune/inflammation/ angiogenesis; c) signal transduction/transcription; d) adhesion/migration; e) metastasis; f) cell cycle regulation,

g) metabolism; h) structural and other genes. Gene selection of this panel takes into account published studies of specific genes altered breast cancer.

Interpretation

Normal tissue from each cancer subject serves as an internal reference when available. For cell lines, where normal DNA is not available, control (normal) female DNA samples are run with each probe set. Quantification, loss or gain of gene loci is determined through a process of normalization. The latter address variations in the surface area of a peak (intensity) encountered due to fluctuations in the assay run such as amount of DNA, ploidy variations, and PCR conditions. Briefly, the peak area for each probe is expressed as a percent of the total surface area of all peaks of a sample in an assay run (**Figures 2 & 3**). Relative copy number for each probe is obtained as a ratio of the normalized value for each locus (peak) of the sample to that of the normal control. A difference is significant only if the ratio is less than 0.7 (loss) or higher than 1.3 (gain). Complete loss or 0 copies is indicated by absence of a peak for that particular locus (illustrated for the homozygous loss of the *CDKN2A*). A relative copy number of 2 is considered normal, 1 or 0 copies is considered loss, and 3 copies or more is considered gain (increased peak height illustrated for *MYC*, **Figure 3**) .

Figure 3   MLPA assay using probe mix 1C
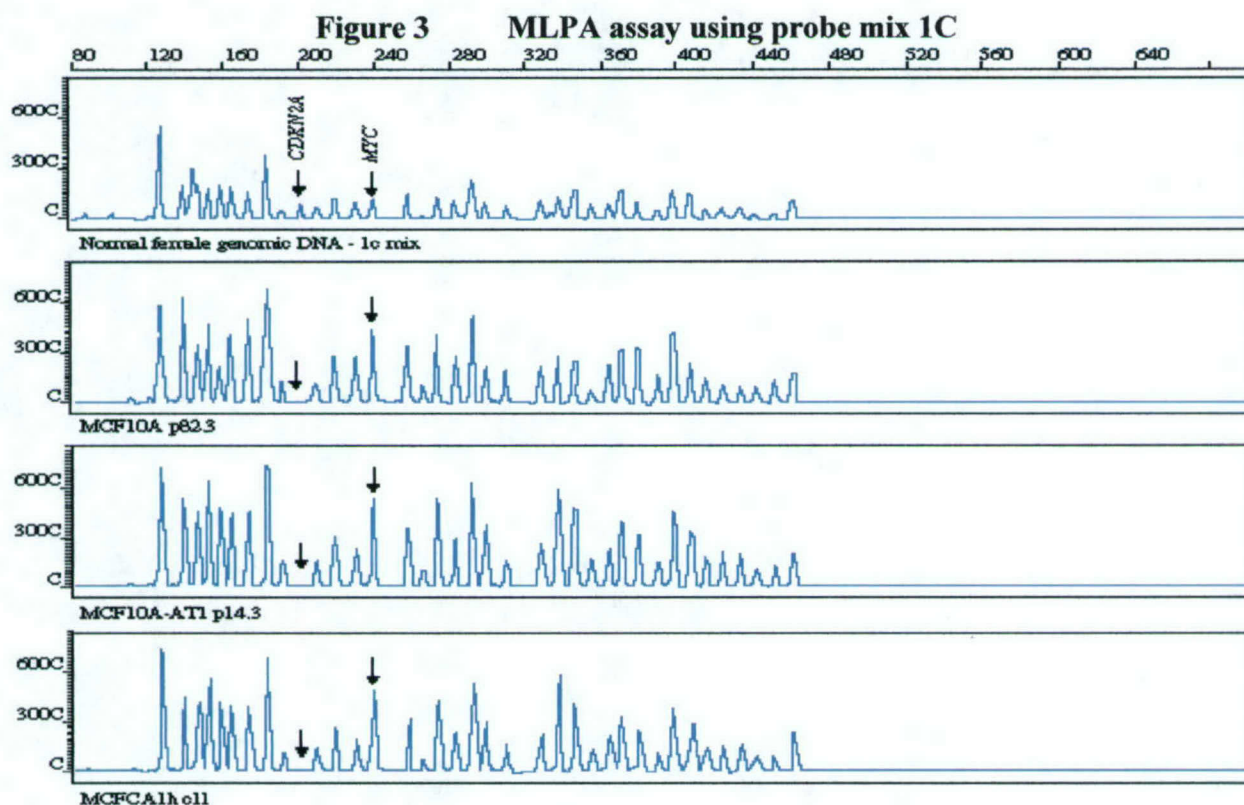
The cytogenetic interpretation for gains and losses has been previously described (10). Briefly, loss of a chromosome or segment was defined as presence of only 1 copy against a near diploid background, or of 1 or 2 copies against a 3N (triploid) or 4N background (near tetraploid). Gain was defined as presence of at least 3 copies against a diploid background, of at least 5 copies against a near triploid background, and of at least 6 copies against a near tetraploid background.

As the amount of DNA available from tumor samples is often very limited and with the advent of laser directed microdissection techniques from very small foci or even single cells, obtaining the desired genetic information becomes crucial and highly dependent on the type of procedures that will permit such analyses.

RESULTS

Ten gene probes were selected with overall p-values <0.1 (Table 4, below) and further tested to show correlation among this specific set of gene probes. The absolute correlation coefficient was in a range of 0 to 1. Gene probes were highly correlated if the absolute correlation coefficient is over 0.70.

Gene probe *TNFRSF1B* at 1p36.3 had a higher copy number for PLCIS compared DCIS with a mean (STD) of 2.32 (0.95) and 1.76 (0.54) respectively, and a p-value =0.03. Gene probe *CASP1* at 11q22.2-q22.3 was the lowest 1.50 (0.55) in LCIS, 1.95 (0.4) in PLCIS, and 2.29(0.46) in DCIS with p-values<0.05 on all the pair-wise comparisons. Four gene probes, *CDH1* at 16q22.1, PDCD8 at Xq25-26, CASP1 at 11q22.2-q22.3 and *PTK2* at 8q24, had a difference in means between PLICS and LCIS with p-values <0.05 (bold). Of these, *CASP1* (11q22.2-q22.3) differed significantly in means among PLCIS, LCIS and DCIS. The correlations among those 10 genes are in a range of low to moderate, which indicate a potential independent predictive ability in a multivariable model.

There were some correlations noted among 5 gene-probes described above in the absolute range of 0.48 between *PTK2* (8q24) and *TNFRSF1B* (1p36.3), and 0.06 between *CASP1* (11q22.2-q22.3), and *TNFRSF1B* (1p36.3).

Table 1: Gene Probe Distribution Amount Tissue Categories

Location	Variable	PLCIS (N= 23)	LCIS (N= 13)	DCIS (N= 21)	p-value PLCIS vs. LCIS	p-value PLCI vs. DCIS	p-value LCIS vs. DCIS
11	LMO2_D01n	2.22 ± 0.81	3.00 ± 0.89	2.05 ± 0.62	0.059	0.478	**0.007**
16q22.1	CDH1_D01n	1.50 ± 0.71	3.17 ± 1.17	1.84 ± 0.69	**<.001**	0.145	**0.002**
Xq25-q26	PDCD8_D01n	1.83 ± 0.38	2.33 ± 0.52	2.00 ± 0.33	**0.019**	0.166	**0.074**
01p36.3	TNFRSF1B_D01n	2.32 ± 0.95	1.83 ± 0.75	1.76 ± 0.54	0.268	**0.033**	0.795
08q11	PRKDC_D01n	2.00 ± 0.58	2.50 ± 0.84	1.95 ± 0.38	0.110	0.758	0.174
08q24	PTK2_D01n	1.95 ± 0.78	3.00 ± 1.26	2.10 ± 0.54	**0.021**	0.486	0.143
11q22.2_q22.3	CASP1_D01n	1.95 ± 0.40	1.50 ± 0.55	2.29 ± 0.46	**0.040**	**0.019**	**0.002**
11q22_q23	BIRC2_D01n	1.84 ± 0.37	2.17 ± 0.41	1.95 ± 0.22	0.083	0.271	0.264
14q13	NFKBIA_D01n	1.74 ± 0.56	2.50 ± 1.22	1.81 ± 0.40	0.193	0.638	0.229
06p21.3	BAK1_D01n	1.50 ± 0.60	1.08 ± 0.79	1.63 ± 0.68	0.093	0.515	0.050

CONCLUSION

Breast cancer is a highly heterogeneous disease, both morphologically and genetically (11). Prognostic marker systems based on single parameters have generally proven inadequate. Thus, multiparametric methods, which rely on many pieces of information, are ideally suited to the grouping of tumor subtypes, identification of specific patterns of disease progression, and in predicting clinical outcomes.

Molecular fingerprints identified from genome wide studies should permit a more robust differential diagnosis of PLCIS aiding in the refinement of this disease phenotype as distinct from other in situ and invasive lesions.

We believe that the molecular strategies when applied to a larger cohort of ins situ breadst carcinomas will be informative in the development and validation of a multivariable genetic blueprint for diagnosis and prognosis of carcinoma in situ. Additionally, the genetic information obtained in our study has the potential to define the molecular basis for tumor heterogeneity, provide preliminary data for exploration of a molecular

staging instrument analogous to the TNM staging system, and may also serve to stratify prognosis and therapy for in situ and invasive breast cancer.

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